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Title: Involvement of Dab1 in APP processing and β-amyloid deposition in sporadic Creutzfeldt-Jakob patients.

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Running title: β-amyloid and prion protein in CJD disease

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ABSTRACT

Alzheimer's disease and prion pathologies (e.g., Creutzfeldt-Jakob disease (CJD)) display profound neural lesions associated with aberrant protein processing and extracellular amyloid deposits. Dab1 has been implicated in the regulation of Amyloid Precursor Protein (APP), but a direct link between human prion diseases and Dab1/APP interactions has not been published. Here we examined this putative relationship in seventeen cases of sporadic CJD (sCJD) *post mortem*. Biochemical analyses of brain tissue revealed two groups, which also correlated with PrP^{sc} types 1 and 2. One group, with PrP^{sc} type 1 showed increased Dab1 phosphorylation, and lower βCTF production with an absence of Aβ deposition. The second sCJD group, which carried PrP^{sc} type 2, showed lower levels of Dab1 phosphorylation and βCTF production, and Aβ deposition. Thus, the present observations suggest a correlation between Dab1-phosphorylation, Aβ deposition and PrP^{sc} type in sCJD.

Keywords: prionopathies, amyloid plaques, Alzheimer's disease, Dab1.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) and Alzheimer's disease (AD) share certain neuropathological and molecular features (Aguzzi and Haass, 2003; Checler and Vincent, 2002; DeArmond, 1993; Ironside and Head, 2008; Price et al., 1993). In TSEs, such as Bovine spongiform encephalopathy (BSE), or Creutzfeldt-Jacob Disease (CJD) in humans, the cellular prion protein (PrP^{c}) is abnormally converted to a protease-resistant form termed PrP^{sc} (Prusiner, 1991; Prusiner, 1998). In Alzheimer's disease, sequential proteolytic cleavage by β- (generating βCTF fragment) and γ-secretases of the amyloid precursor protein (APP) leads to an extracellular overproduction of β-amyloid (Aβ) peptides. They consist of 40-42 amino acids and comprise the senile plaques characteristic of such proteinopathies (Mattson, 2004; Selkoe, 2001). Deposition of both PrP^{sc} and Aβ in the central nervous system (CNS) results in neurodegeneration. Moreover, the coexistence of Aβ and PrP^{sc} amyloid deposits in affected brains, although controversial, has been widely reported (Barcikowska et al., 1995; Debatin et al., 2008; Ferrer et al., 2001; Hainfellner et al., 1998; Leuba et al., 2000; Tsuchiya et al., 2004). In addition, a recent study indicates that PrP^{c} may participate in the removal of Aβ oligomers, which would implicate PrP^{c} in AD (Charveriat et al., 2009).

CJD is the most common human prion disease; it is classified as sporadic (sCJD), familial (fCJD), iatrogenic (iCJD) and variant (vCJD) (Glatzel et al., 2003). Although sCJD is rare, with an incidence of 0.6-1.2 per million, it accounts for about 85% of all recognized human cases of prion disease (Brandel et al., 2000; Hill et al., 2003). Two predominant PrP^{sc} types have been identified, based on the gel mobility of the PrP^{sc} fragments resistant to proteinase K (PK) treatment, and they are associated with different CJD phenotypes (Cali et al., 2006; Gambetti et al., 2003; Monari et al., 1994; Petersen et al., 1994). Parallel to this, *PRNP* polymorphism at codon 129

(Met^{129}→Val) modulates sensitivity to CJD, and methionine homozygosis is a risk factor for sporadic and variant CJD (Collinge, 1999; Ladogana et al., 2005; Palmer et al., 1991). However, conflicting results have been reported about the relationship between polymorphism at codon 129 and the pathological and clinical features of AD (Hooper and Turner, 2008; Poleggi et al., 2008) for reviews) although a systematic meta-analysis of AD genetic association studies revealed *PRNP* as a susceptible gene ((Bertram and Tanzi, 2008) for review).

APP processing is highly regulated. Disabled-1 (Dab1) is a cytosolic adaptor protein that is phosphorylated by members of the Src family of tyrosine kinases (SFK) in response to extracellular molecules during neural development (Howell et al., 1999b). Several studies implicate Dab1 in APP processing (Hoe et al., 2006; Parisiadou and Efthimiopoulos, 2007; Trommsdorff et al., 1998). We have recently examined whether APP processing and Dab1 phosphorylation are involved in cellular changes mediated by the synthetic peptide PrP(106-126) *in vitro* and by PrP^{sc} *in vivo* in 263K scrapie-inoculated hamsters (Gavin et al., 2008). PrP(106-126) induces APP accumulation in cultured neurons (Gavin et al., 2008; White et al., 2003) but also reduces levels of βCTF and Aβ production (Gavin et al., 2008). However, these data need further corroboration in human CJD patients. With this in mind, in the present study we analyze the putative correlation between PrP^{sc} type (1 and 2), APP processing and Aβ plaque formation in a series of sCJD patients. Our findings indicate that Dab1 contributes to β-amyloid deposition in sCJD brains.

PATIENTS AND METHODS

Cases

Seventeen brains were excised from the cadavers of sCJD patients between 3h and 8h *post mortem.* Brain tissue was immediately prepared for morphological and biochemical studies. The main clinical and pathological characteristics are summarized in Table 1. For biochemical studies, samples of the parietal cortex were frozen in liquid nitrogen and stored at -80º C until use. For neuropathological diagnosis, 4% formalin-fixed, formic acid-treated samples were embedded in paraffin. The neuropathological study was carried out on de-waxed 4-µm-thick paraffin sections of the frontal (area 8), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior gyrus cinguli, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate putamen and globus pallidus; medial and posterior thalamus; hypothalamus; Meynert nucleus; amygdala; midbrain (two levels), pons and bulb; and cerebellar cortex and dentate nucleus. The sections were stained with haematoxylin and eosin, Klüver Barrera, and, for immunohistochemistry to glial fibrillary acidic protein (Dako, Glostrup, Denmark, dilution 1:250), CD68 for microglia (Dako, dilution 1:100), β-amyloid (Boehringer, Ingelheim, Germany, dilution 1:50), tau AT8 (Innogenetics, Ghent, Belgium, dilution 1:500), αB-crystallin (Abcam, Cambridge, MA, USA, dilution 1:100), α-synuclein (Millipore, Buillerica, MA, USA, dilution 1:500), ubiquitin (Dako, dilution 1:200), and PrP, (clone 3F4, (Dako, dilution 1:1000) with and without proteinase K pre-incubation). Immunohistochemical controls included omission of the primary antibody or incubation with non-specific immunoglobulins.

PrP typing

Samples of the frontal cortex (0.1 g) were homogenized in a bounce homogenizer containing 9 volumes of ice-cold lysis buffer (ICLB) (100 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, pH 6.9).

Homogenates were centrifuged at 2,000x *g* for 5 min. The resulting supernatant (100 µl) was incubated with 100 µg/ml of Proteinase K for 1 h at 37ºC. The reaction was stopped by the addition of 5 µl of PMSF; the solution was then mixed with 2X sample buffer and boiled at 96º C for 5 min. 15 µl of the different samples was loaded side by side with the corresponding total homogenates, and electrophoresed in a 12% SDS-PAGE gel. Electrophoresed gels were transferred to nitrocellulose membranes, which were subsequently blocked with 5% non-fat dried milk in 0.1M Tris-buffered saline (TTBS) and incubated with the 3F4 mouse monoclonal anti-PrP antibody at 4ºC overnight. The membranes were washed in TTBS, incubated with the secondary anti-mouse antibody and developed with the ECL system. In PrP^{sc} type 1 the PK resistant domain has a gel mobility of about 21 KDa and commonly has its N-terminus, corresponding to the main PK cleavage site, at residue 82. In PrP^{sc} type 2, the corresponding PK-resistant domain migrates on gel to approximately 19 KDa, and its N-terminus usually starts at residue 97 (Parchi et al., 2000)(Fig. 1).

Codon 129 genotyping

PRNP sequencing was carried out in every case. The putative amino acid polymorphism at codon 129 of the PRNP gene, a methionine (M) to valine (V) substitution resulting from an adenine to guanine transition was evaluated. We used allele-specific oligonucleotide (ASO) hybridization to amplified genomic DNA as described in Owen *et al.* (Owen et al., 1990). No mutations in *PRNP* were seen. Homozygosity at codon 129 was examined in every case.

Immunoprecipitation and Western immunoblotting

Tissue samples were labeled as A, B, C, etc, after diagnosis in the Neuropathological institute without any indication of the PrP^{sc} type or 129 genotyping. Thus samples were processed for Immunoprecipitation, Western blotting and

densitometry in “blind” conditions. After the densitometrical study (see below) each sample was identified and the data were correlated. Thus, samples of the parietal cortex (0.1 g) were homogenized in a glass homogenizer containing nine volumes of ICLB and then centrifuged. The resulting supernatant was normalized for protein content. After that, one fraction was mixed with twice-concentrated Laemmli sample buffer and boiled at 96º C for 5 minutes, and 30 µg of each sample was processed for immunoblotting. The second fraction was used for determination of Dab1 phosphorylation levels. Thus, 1000 µg total protein was incubated with αDab1 (Exalpha Biologicals, Watertown, MA, USA) at 4 µg in 500 µl total volume overnight at 4º C. Afterwards, immune complexes were precipitated using Protein-G-Sepharose (Amersham-Pharmacia Biotech; GE Healthcare, Barcelona, Spain) at 4º C for 90 minutes. After centrifugation, proteins were eluted in twice-concentrated Laemmli sample buffer at 96º C for 5 minutes, followed by 10% SDS-PAGE and immunoblotting using the anti-phosphotyrosine 4G10 (Upstate Biotechnology Inc., Lake Placid, NY, USA). Membranes were reprobed with ab7522 antibody for total Dab1 detection (Abcam, Cambridge, MA, USA). In parallel experiments, the βCTF fragment was detected using the ab2073 antibody (Abcam) and re-probed with an antibody against Actin (Sigma, Saint Louis, MO, USA). For immunoblotting, proteins were electrotransferred to nitrocellulose membranes for 6 hours. Membranes were then blocked with 3% BSA or 5% non-fat milk in 0.1M TTBS for 2 hours and incubated overnight in 0.5 % blocking solution containing primary antibodies. After incubation with peroxidase-tagged secondary antibodies (diluted 1:2000), membranes were revealed by ECL-plus chemiluminescence Western blotting kit (Amersham-Pharmacia Biotech). In our experiments, each nitrocellulose membrane was used to detect both phosphorylated and total Dab1 levels or βCTF and Actin. To perform this sequential incubation, membranes were incubated in 25 ml of stripping solution (2% SDS, 62.5 mM Tris pH 6.8, 100 mM 2-mercaptoethanol) for 30 min at 65º C

and then extensively washed before reincubation with blocking buffer and antibodies for re-blotting.

Densitometry and statistical processing

For quantification, developed films were scanned at 2,400 x 2,400 dpi (i800 MICROTEK high quality film scanner) and the densitometric analysis was performed using Quantity One Image Software Analysis (Biorad). Statistical analysis of the resulting data was performed using STATGRAPHICS plus 5.1 and Origin 8™ programs with the ANOVA test. Asterisks in the histograms indicate the following *p* values of significance: (*) $p < 0.05$; (**) $p < 0.01$.

RESULTS

Criteria for the neuropathological, molecular and phenotypic diagnosis of CJD used in the present series are those currently accepted and detailed elsewhere (Budka, 2003; Ironside and Head, 2008; Parchi et al., 1999; Parchi et al., 2009). Neuron loss, spongiform degeneration, astrocytic gliosis and microgliosis involving the cerebral neocortex, striatum and cerebellum, occurred in every case. Synaptic-like PrP^{sc} deposits were also found in the cerebral cortex and striatum in every case. In addition, PrP^{sc} plaques were common in three Val/Val (VV) cases. No Lewy bodies or αB-crystallin-immunoreactive ballooned neurons were seen in any case. Ubiquitin deposition was restricted to scattered small dots in the cerebral cortex and white matter. Neurofibrillary tangles were absent except for a very few neurons in the entorhinal cortex in a few cases (corresponding to stages I and II of Braak; CJD9; CJD12, CJD14, CJD17 cases (Table 1). β-Amyloid deposition in the parietal occurred in seven cases, (Table 1). This region was representative of other cortical areas, particularly the temporal and orbital cortices, as revealed by immunohistochemistry. The β-amyloid load was quantified by counting the number of plaques in five squares of the cerebral cortex per section, in four non-consecutive sections per case at x200 magnification (using a x40 oil-immersion objective). Data were scored as follows: score 0 = absence of plaques; score + = less than five diffuse plaques per field; score ++ = more than five diffuse plaques and presence of core plaques per field. There was no correlation between the presence of neurofibrillary tangles and β-amyloid plaques in these series.

Analysis of Dab1 phosphorylation revealed two groups of sCJD cases

Dab1-phosphorylation was measured by immunoprecipitation (Gavin et al., 2008). Total Dab1 protein levels were also measured by Western blot using the ab7522

antibody. In these cases, the amount of actin was used as an internal control of protein loading. In the first experiments, densitometric analysis of phosphorylated Dab1 (85 KDa band) showed two groups of sCJD cases. We divided the samples into two groups for further analysis (Fig. 2A-D). One group of eight sCJD cases showed higher Dab1 phosphorylation (0.85 ± 0.13; mean ± sem) than controls (0.59 ± 0.17), and the other nine cases did not (0.62 ± 0.16) (Fig. 2B). These results correlate with total levels of Dab1 in protein samples (0.74 ± 0.11 (sCJD group 1); 0.93 ± 0.16 (controls) and 0.86 ± 0.12 (sCJD group 2) (Fig. 2D). Further identification of the PrP^{sc} type in processed samples showed that these two groups correlated with PrP^{sc} types 1 and 2 respectively.

βCTF production and Aβ deposition in sCJD

We measured the contents of the product of the β-secretase activity in sCJD samples (Fig. 3) and correlate the data with phosphorylated Dab1 levels (Fig. 4) and the presence of Aβ deposits in parallel histological sections (Fig. 5). Western blotting analysis of sCJD samples revealed that the first group of sCJD cases showed lower levels of βCTF (1.48 ± 0.37) than controls (1.93 ± 0.36). In contrast, the second sCJD group did not (1.65 ± 0.28) (Fig. 3). Next we plotted in a double-Y graph the levels of βCTF and pDab1 for each sCJD sample and controls (Fig. 4). PrP^{sc} type 1 samples showed high Dab1 phosphorylation and low βCTF production compared to PrP^{sc} type 2 samples and controls. In contrast, these clear differences were not observed between PrP^{sc} type 2 and control samples (Fig. 4).

The presence or absence of β-amyloid was examined in the parietal cortex of every sCJD case as described above. Histological samples are illustrated in Fig. 5A-C.

No β-amyloid immunoreactivity was seen in brain homogenates of cases with no β-amyloid plaques (e.g. case CJD4) (not shown). The presence of Aβ plaques in the parietal cortex correlated with the presence or absence of Dab1 phosphorylation. Aβ deposits were absent in the first sCJD group but frequent in the second.

Correlation between codon 129 polymorphism with PrP^{sc} type and Aβ deposits in sCJD groups

Lastly, we correlated codon 129 polymorphims of every sCJD case with the biochemical data, the histopathological study (Aβ deposits) and the PrP^{sc} typing. Most members of the first sCJD group (n = 7), with PrP^{sc} type 1, had no Aβ deposits and Met/Met (MM) polymorphism. Only one case out of the eight in the first sCJD group displayed a VV polymorphism. In contrast, the second sCJD group, with PrP^{sc} type 2, showed increased numbers of Aβ deposits (ranked from + to ++) but only in VV (n = 5) and Met/Val (MV) (n = 1) polymorphism (Table 1). In conclusion, PrP^{sc} type 1 correlated strongly with MM polymorphism, high Dab1 phosphorylation, low βCTF production and the absence of Aβ deposits. In contrast, PrP^{sc} type 2 did not fully correlate with a clear polymorphism, although MM cases with PrP^{sc} type 2 also lacked Aβ deposits, in contrast to MV and VV polymorphisms.

DISCUSSION

In the present study, we examined whether APP processing and Dab1 phosphorylation correlate with the neuropathological changes, PrP^{sc} typing and 129 genotype observed in sCJD cases. As commercial antibodies against phospho-Dab1 do not react in post-mortem tissue, we assessed the degree of Dab1 phosphorylation by immunoprecipitation and Western blot. In addition, to avoid mixed responses due to coexisting prion species in brain regions (Polymenidou et al., 2005), we chose to study the parietal cortex since in this area it is relatively easy to differentiate between type 1 or type 2 PrP^{sc}. We determined that the presence of PrP^{sc} type 1 in sCJD patients correlated with MM polymorphism, low βCTF production and the absence of Aβ deposits. In contrast, PrP^{sc} type 2 did not correlate with a clear polymorphism, although patients MM carring PrP^{sc} type 2 also lacked Aβ deposits, in contrast to other polymorphisms. In addition, PrP^{sc} type 1 cases showed higher Dab1 phosphorylation than controls, and PrP^{sc} type 2 cases did not. Taken together these results suggest a correlation between Dab1-phosphorylation, Aβ deposition and PrP^{sc} type in sCJD.

To date, the molecular mechanisms underlying prion pathology remain to be fully clarified (e.g., see (Aguzzi et al., 2008; Nicolas et al., 2009; Weissmann, 2004) for reviews). Most intracellular mechanisms in proteinopathies with amyloid deposits are expected to be similar (e.g., (Gaggelli et al., 2006)). Therefore, some crosstalk points between these PrP^{c} functions and Aβ deposition have been described in recent years, although controversial. For example, PrP^{c} binds Aβ oligomers, thus decreasing Aβ deposition (Lauren et al., 2009). In addition, Hooper and Turner described that PrP^{c} inhibits BACE1-dependent APP cleavage (Hooper and Turner, 2008). In contrast, other authors have indicated that PrP^{c} increases Aβ deposition (Schwarze-Eicker et al., 2005)

or that Aβ levels increase in an AD mouse model after prion inoculation (Baier et al., 2008).

On the other hand, a recent study has described a relation between PrP^{sc} type and Aβ deposition in the cerebellum of sCJD with older age at disease onset and long disease duration (Debatin et al., 2008). Moreover, sCJD patients with abundant Aβ harbored lower amounts of PrP^{sc} in the cerebellum (Debatin et al., 2008). This is important, as Aβ deposition generally increases with the age of the individual. In our study, the mean age of cases with higher Aβ deposits was 68 years, whereas the mean age of cases without Aβ deposition (excluding CJD1) was 66 years. In both groups, individual cases were relatively young (i.e. 52 years) or relatively old (i.e. 82 years). An unusual case is represented by CJD1 who was 25 years old. This last case is consistent with other studies indicating that sCJD also affect young patients (Budka et al., 2003, Ironside and Head 2008).

Our study points to an intracellular adaptor (Dab1) as a putative link between PrP^{sc} and Aβ-deposition. Dab1 was initially described as a cytosolic adaptor protein phosphorylated by members of the Src family of tyrosine kinases (SFK) in response to extracellular signals and by oxidative stress *in vitro* (e.g., (Howell et al., 1999a)). In addition, Dab1 has been implicated in APP-processing (e.g., (Trommsdorff et al., 1998)). Our results corroborate previous findings of our group, that Aβ production is lower in *mdab1*-deficient neuronal cultures (Gavin et al., 2008). We thus conclude that PrP^{sc} may affect Dab1 phosphorylation and Aβ production. PrP^{sc} inoculation gives rise to a wide range of molecular responses in infected brains (Xiang et al., 2004) including in oxidative stress (Choi et al., 1998; Martin et al., 2007; Yun et al., 2006), which is also described in sCJD patients (Freixes et al., 2006; Pamplona et al., 2008; Petersen et al.,

2005). Indeed, sCJD patients with PrP^{sc} type 1 and MM polymorphism at codon 129 are characterized by faster evolution and decreased life expectancy compared with PrP^{sc} type 2 with other polymorphisms at codon 129 (Uro-Coste et al., 2008). These data suggest that oxidative abnormalities in sCJD patients with PrP^{sc} type 1 may alter Dab1 function, impairing normal APP processing and decreasing Aβ production. However, we cannot exclude that other precesses such us impairment of copper homeostasis (Schoch et al., 2006), or unknown new roles of PrP^{c} (Lauren et al., 2009) may alters Aβ deposition in sCJD brains. These putative scenarios must be taken in account in further study. However, our present results suggest that Dab1 is involved in both sCJD and AD.

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FIGURE LEGENDS

Figure 1.

Patterns of PrP^{sc} type 1 and type 2 (PK: proteinase K pre-treatment). Three examples of PrP^{sc} processing are illustrated. Every sample is run in parallel with a negative control (lane 1), a typical case of PrP^{sc} type 1 (lane 2), a typical case type 2 (lane 3) and the case problem (lane 4).

Figure 2.

Example of Western blot determination of pDab1 **(A-B)** and total Dab1 protein levels **(C-D)** in sCJD cases. sCJD cases were categorized as described above. Protein samples from different groups of sCJD (first and second groups) are shown. **B)** The densitometric results are shown. Each data item corresponding to a sCJD case is displayed in the histograms. In addition, the mean and SEM in each group is also shown. A significant increase in the pDab1/Dab1 ratio is observed in the first group of sCJD cases compared to the second sCJD group and controls. **C-D)** Parallel determination of total Dab1 levels in the same sCJD protein samples. The increased phosphorylation of Dab1 in the first sCJD cases correlates with decreased levels of total protein. Each dot corresponds to a single case. Asterisks indicate significant differences between sCJD groups and controls in **(B and D)**. * $p < 0.05$; ** $p < 0.01$ (ANOVA test).

Figure 3.

Example of Western blotting determination of βCTF **(A-B)** in sCJD cases compared to controls. sCJD cases were categorized as described above. Decreased levels of βCTF can be seen in the first sCJD group compared to controls. **B)** Histograms showing the densitometric study as in Figure 2. Each dot corresponds to a single case. Asterisks

indicate significant differences between sCJD groups and controls. * $p < 0.05$; (ANOVA test).

Figure 4

Double-Y graphs illustrating the densitometric results of pDab1/Dab1 ratio (left Y axis) and CTFβ levels (blue Right Y axis) for each case (X Axis). Each dot/square corresponds to a single case. Values of pDab1/Dab1 (black squares) and CTFβ (blue circles) have been linked with a line and the area (grey for pDab1/Dab1 and violet for CTFβ) has been completed for each patient group. Notice the clear differences in the distribution of the grey and violet areas between the 1st and the 2nd group of sCJD cases and controls.

Figure 5.

Low power photomicrographs illustrating examples of amyloid plaques in some of the sCJD cases used in the present study after Aβ immunocytochemistry. **A)** no plaques (score 0); **B)** a few diffuse plaques (score +); **C)** many diffuse plaques, some neuritic plaques (score ++). See Results for details. Scale bar A = 500 μm pertains to B-C.